# AMPK

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# AMPK: an energy-sensing pathway with multiple inputs and outputs

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## Summary

The AMP-activated protein kinase (AMPK) is a key regulator of energy balance expressed ubiquitously in eukaryotic cells. Here, we review the canonical adenine nucleotide-dependent mechanism that activates AMPK when cellular energy status is compromised, as well as other, non-canonical activation mechanisms. Once activated, AMPK acts to restore energy homeostasis by promoting catabolic pathways, resulting in ATP generation, and inhibiting anabolic pathways that consume ATP. We also review the various hypothesis-driven and unbiased approaches that have been used to identify AMPK substrates, which have revealed substrates involved in both metabolic and non-metabolic processes. We particularly focus on methods for identifying the AMPK target recognition motif, and how it can be used to predict new substrates.

## AMPK – subunit structure and regulation

The AMP-activated protein kinase (AMPK) is a key sensor of cellular energy status present in essentially all eukaryotic cells, where it occurs as heterotrimers comprising catalytic α subunits and regulatory β and γ subunits [1-3]. Genes encoding at least one of these subunits are found in the genomes of essentially all eukaryotes, while mammals have genes encoding multiple isoforms (α1, α2; β1, β2; γ1, γ2, γ3). AMPK heterotrimers are normally only significantly active after phosphorylation of a conserved threonine residue within the activation loop of the α subunit kinase domain (Thr172 in rat α2 [4]; the numbering may differ in other species). Mammalian AMPK is activated through binding of 5'-AMP by three complementary effects (Fig. 1): i. promotion of Thr172 phosphorylation by upstream kinases; ii. inhibition of Thr172 dephosphorylation by protein phosphatases; and iii. allosteric activation. Although allosteric activation is only triggered by binding of AMP, effect #1 (promotion of Thr172 phosphorylation [5]) and #2 (inhibition of Thr172 dephosphorylation [6, 7]) can be mimicked by ADP. Since ATP antagonizes these effects, AMPK acts as a sensor of cellular AMP:ATP and ADP:ATP ratios, both of which increase during cellular energy stress (although the changes in AMP:ATP are always larger due to the adenylate kinase reaction [7]). AMPK can sense small changes in AMP even in the presence of concentrations of ATP two to three orders of magnitude higher [7, 8].

In this review, we will discuss recent studies of the molecular mechanisms by which AMPK is activated by the canonical inputs AMP and ADP (introduced above), as well as by the non-canonical inputs that are being increasingly recognized. We also discuss recent approaches aimed at establishing the full complement of downstream targets that are phosphorylated in cells when AMPK is activated.

## Canonical and non-canonical inputs into the AMPK system

### *Canonical inputs - adenine nucleotide binding to the AMPK-γ subunit*

AMPK senses changes in AMP through its direct binding to the γ subunit. AMPK-γ subunits in all species contain four tandem repeats of sequence motifs known as cystathionine β-synthase (CBS) repeats. These also occur in a small number of other proteins in the human genome, although usually as just two tandem repeats. In many cases, each tandem pair of repeats bind a regulatory

adenosine-containing ligand, such as ATP or S-adenosyl methionine, in the cleft between the repeats [9]. In AMPK-γ subunits, the four repeats assemble into a disc-like shape with one repeat in each quadrant, generating four potential nucleotide-binding sites that are numbered according to which repeat binds the ribose ring of each nucleotide. Of these, site 2 appears to be always vacant, while site 4 is thought to contain only a permanently-bound AMP [10]. Although the latter view has been challenged [11], this would leave sites 1 and 3 as the sites where AMP, ADP and ATP bind in competition.

A crystal structure [8] of the human α1β2γ1 complex containing several bound ligands (AMP, the kinase inhibitor staurosporine and the glycogen mimetic β-cyclodextrin) is shown in Fig. 2; it is similar to previous structures of α2β1γ1 [12] and α1β1γ1 [13] complexes. The kinase domain on the α subunit (α-KD), containing the small N-terminal and larger C-terminal lobes of a typical protein kinase, is immediately followed by the autoinhibitory domain (α-AID), which is so-called because KD:AID constructs are 10-fold less active than those containing the KD alone [14-16]. Structures of KD:AID constructs from fission yeast [17], and human α1 [8], reveal the α-AID to be a compact bundle of three α-helices that inhibits the kinase by binding of its helix α3 to the N- and C- lobes of the α-KD, on the opposite surface to the active site (Fig. 3A). In all structures of active kinase domains, four hydrophobic side chains known as the *regulatory spine* are stacked in alignment, indicating the correct disposition of active site residues [18]. In the inactive KD:AID structure shown in Fig. 3A, these residues (shown in white, red, magenta and blue) are not aligned. By contrast, in the structures of active AMPK heterotrimers, the α-AID has rotated away from the α-KD, with its α3 helix now interacting with the second CBS repeat of the γ subunit instead (Fig. 2), and the side chains of the regulatory spine are now aligned (e.g. Fig. 3B).

The α-AID is connected to the C-terminal domain of the α-subunit (α-CTD) by a critical region of extended polypeptide termed the α-linker. In the view of Fig. 2 this linker (shown in space-filling representation in blue, red and magenta) wraps around the front face of the γ subunit. It contains two conserved motifs, termed α-RIM1 and α-RIM2 *(RIM = regulatory subunit interacting motif)* [19]. In structures of active heterotrimers, α-RIM1 (in blue in Fig. 2) binds to the surface of the γ subunit containing the vacant site 2, while α-RIM2 (in magenta) interacts with site 3 containing bound AMP. This tight association of the α-linker with the AMP-bound form of the γ subunit is proposed to cause the observed rotation of the α-AID away from the α-KD, thus explaining how

binding of AMP at site 3 causes allosteric activation [12, 19]. This model requires that binding of ATP at site 3 would not allow the same interaction with α-RIM2. Supporting this, the interaction between an α-AID:linker fragment and a construct containing the γ subunit was shown by luminescence energy transfer to be enhanced by AMP binding, but decreased by ATP binding [8].

Recent experiments with a novel AMPK activator suggest that the ability of AMP analogs to protect against Thr172 dephosphorylation (effect #2) is also due to binding at site 3. C13 (see Fig. 4A) is a phosphonate diester that is taken up into cells and converted by cellular esterases to C2, a potent AMP analog [20]. In cell-free assays, C2 promoted effects #2 (protection against Thr172 dephosphorylation) and #3 (allosteric activation) using α1-containing complexes, but in α2-containing complexes, it was only a partial allosteric agonist compared to AMP, and failed to protect against Thr172 dephosphorylation. However, the full effects could be transferred to α2-containing complexes merely by replacing part of the α-linker from α2 (including α-RIM2) with the equivalent region from α1 [21]. Since α-RIM2 contacts site 3, but no part of the α-linker contacts sites 1 or 4 (which are on the opposite face of the γ subunit), binding of C2 at site 3 seems to be crucial for effect #2 as well as #3. This leaves open the question of the functions, if any, of AMP binding at sites 1 and 4.

What is the exact mechanism for effect #2 (protection against Thr172 dephosphorylation)? Current structures of the heterotrimer, which are in active conformations with AMP bound in site 3, can be divided into two major regions termed the "catalytic module" (upper left in Fig. 2) and "nucleotide-binding" module (lower right in Fig. 2), with Thr172 located in the cleft between them. The α-linker can be regarded as a flexible connector linking these two modules, and its release from the γ subunit when ATP replaces AMP at site 3 may allow the catalytic and nucleotide-binding modules to move apart, increasing the accessibility of Thr172 to phosphatases, thus inducing dephosphorylation [6]. This movement would also allow the α-AID to move back into its inhibitory position behind the α-KD, thus reversing allosteric activation. In support of this model, measurements of small angle X-ray scattering [22], and luminescence energy transfer [8] both suggest that heterotrimers adopt less compact conformations when ATP, rather than AMP, is bound.

The explanation for effect #1 (promotion of Thr172 phosphorylation by LKB1) remains unknown, although an intriguing recent proposal is that AMP binding causes AMPK to co-localize

with LKB1 due to their mutual interactions with the scaffold protein axin, which in turn binds to LAMTOR1 at the surface of the lysosome [23, 24]. However, effect #1 can also be observed on reconstitution of highly purified LKB1 and AMPK [7], suggesting that it does not strictly require these additional components.

### *Non-canonical inputs - activation by ligands that bind between the α and β subunits*

Many screens of candidate molecules, as well as unbiased screens, have been conducted in the hunt for AMPK activators that might have therapeutic potential. A small selection of known activators is shown in Fig. 4. One class (Fig. 4A), including C13 [20] and 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) [25], are pro-drugs converted by cellular enzymes into AMP analogs that bind to the γ subunit. A second class (Fig. 4B) is exemplified by A-769662 [26], which does not bind at the AMP-binding sites even though, like AMP, it causes both allosteric activation and protection against Thr172 dephosphorylation [15, 27]. Binding of A-769662 involves the β-subunit carbohydrate-binding module (β-CBM), which is related to non-catalytic domains found in enzymes that metabolize glycogen or starch, and has been shown to cause binding of AMPK to glycogen particles within cells [28, 29]. In the structure shown in Fig. 2, the synthetic oligosaccharide β-cyclodextrin was bound at the presumed glycogen-binding site. In other recent structural analyses [12, 13], A-769662 or another activator, 991 (also known as ex229 [30]), was bound at the opposite surface of the β-CBM, in the cleft between it and the α-KD N-lobe; this cleft is labeled in Fig. 2 although unoccupied. A-769662 and 991 are synthetic molecules, but the natural plant product and AMPK activator, salicylate, was suggested to bind at the same site [31]; this was recently confirmed by a crystal structure containing bound 5-iodosalicylate [13]. Salicylate (in the form of willow bark extracts) has been used as a medicine since ancient times, with acetyl salicylic acid (aspirin) being a synthetic derivative that is rapidly broken down to salicylate *in vivo*. However, this leaves open the question as to whether there is a naturally occurring metabolite in mammals that regulates AMPK by binding this site. Although such a metabolite has not yet been found, the site has been referred to as the allosteric drug and metabolite (ADaM) binding pocket [32].

### *Non-canonical inputs – activation by the $Ca^{2+}$/CaMKKβ pathway*

Hormones that increase intracellular $Ca^{2+}$ activate AMPK via phosphorylation of Thr172 by the calmodulin-dependent protein kinase CaMKKβ (Fig. 1) [33-35]. This represents an alternate, $Ca^{2+}$-dependent pathway for AMPK activation that is independent of changes in adenine nucleotides and is therefore considered to be non-canonical. However, because AMP binding inhibits Thr172 dephosphorylation, the two pathways can act synergistically [36].

### *Indirect mechanisms for AMPK activation*

Most other activators (Fig. 4C) activate AMPK indirectly by inhibiting ATP synthesis, thus increasing intracellular AMP:ATP and ADP:ATP ratios [37]. These include the glycolytic inhibitor 2-deoxyglucose, the anti-diabetic drugs metformin and phenformin, and many natural plant products that are either used in traditional medicines or are being tested for health-promoting activities (e.g. berberine [38], arctigenin [39], and resveratrol [40]). Another interesting activation mechanism is displayed by the tetrahydrofolate analogs pemetrexed and methotrexate, used for treatment of cancer or inflammatory disorders such as rheumatoid arthritis. These analogs inhibit tetrahydrofolate-utilizing enzymes, including the transformylase that catalyzes the first step in the metabolism of ZMP, the phosphorylated form of AICAR, to purine nucleotides (although inhibition by methotrexate is most likely secondary to inhibition of dihydrofolate reductase [41]). They therefore cause accumulation of cellular ZMP, which binds to the γ subunit and activates AMPK (Fig. 4D) [41, 42].

## Outputs: identification of downstream targets

Once AMPK is activated, it acts to restore energy homeostasis by activating catabolic pathways that generate ATP and inhibiting anabolic pathways that consume ATP. However, since the great majority of cellular processes consume energy and most are coupled to ATP hydrolysis, there is no reason why processes switched off by AMPK should be restricted to metabolic roles. There has therefore been a need to understand how AMPK recognizes its target phosphorylation sites, and to develop methods to screen for, and even predict, novel downstream targets. This will form the central theme of the remainder of this review, and some of the methods used are illustrated in Fig. 5.

### *Initial hypothesis-driven approaches to determine the AMPK recognition motif*

Sequencing of the first few sites phosphorylated by AMPK led to the identification of conserved residues close to the phosphoacceptor site (P), including basic residues at P-3 and/or P-4 and hydrophobic residues at P-5 and P+4, which were proposed to be important in recognition. This was confirmed by making replacements within the *SAMS* peptide [43], which was based on the sequence around Ser79 on rat acetyl-CoA carboxylase-1 (ACC1) and was the first peptide substrate for AMPK [44]. This study also showed that AMPK would phosphorylate threonine, although serine was preferred (this preference for serine is a feature of serine/threonine kinases in general). A follow-up study utilized a specially designed peptide sequence (*AMARAASAAALA*) where all residues other than the serine and the critical positions at P-5, P-3, and P+4 were alanine. This study showed that the mammalian, budding yeast, and higher plant orthologs of AMPK had very similar specificities, and that the *position* as well as the chemical nature of the P-5, P-3 and P+4 residues was crucial, although the basic residue could be at P-4 or P-3 [45]. Another approach used a bacterially expressed peptide containing 34 residues around the Ser79 site on rat ACC1 as the substrate. A model for the binding of this sequence to the kinase domain was generated by homology to structures of closely related kinases. By making complementary mutations of kinase and substrate, and analyzing their effects on phosphorylation kinetics (Fig. 5A), this model was validated [46]. An additional positive determinant identified in this study was a basic residue at P-6, although not all known AMPK substrates contain this. This study also suggested that the sequence N-terminal to P-5 in ACC1, in which conserved hydrophobic residues occur every 3-4 residues (at P-5, P-9, P-13 and P-16), form an amphipathic α-helix that fits into a hydrophobic groove within the C lobe of the α-KD. Interestingly, a partial structure of another AMPK target, HMG-CoA reductase [47], revealed that residues from Gly860 to Arg871 (corresponding to P-12 to P-1 with respect to the phosphorylation site, Ser872) do form such an α-helix. However, this amphipathic helix cannot be essential for target recognition, because it is not present in the peptide substrates, or in the liver or muscle isoforms of glycogen synthase, two well-validated targets for AMPK where the P-6 residues actually form the N-termini of the mature proteins. These α-helices should therefore be regarded as docking sites that enhance the affinity of the kinase-substrate interaction, rather than as essential determinants for substrate recognition.

### *Unbiased approaches to determine the recognition motif*

The studies described in the previous section were built on hypotheses about substrate recognition derived from sequences of a relatively small number of known targets. More recently, unbiased screens to identify the preferred AMPK recognition motif have been developed. The first was a peptide library approach using spatially arrayed sets of peptide mixtures [48], a method originally developed for studies of other kinases [49]. Each mixture contained a fixed amino acid at a given position relative to a central fixed serine/threonine phosphoacceptor, with mixtures at all other positions (Fig. 5B). From the relative amount of phosphate incorporated into each mixture, a measure of selectivity for and against individual amino acids at each position was obtained. Gratifyingly, the results yielded a recognition motif remarkably similar to the earlier approaches, with hydrophobic side chains being preferred at P-5 (especially M or L) and P+4 (I, L, M or F), and basic side chains (R>K) at the P-4 or P-3 position; a preference for polar side chains at P+3 was also noted [48]. One drawback of this approach is that it is not feasible to study the effects of amino acids more than about five residues away from the phosphoacceptor, because the peptide mixtures become too degenerate.

A second method, represented in Fig. 5C, involved identification of large numbers of direct targets in intact cells and allowed analysis beyond the P-5 and P+4 positions [50]. This approach utilized a method where the ATP binding pocket of the kinase was enlarged by mutation to accept a bulky ($N^6$-phenethyl) derivative of ATP containing γ-thiophosphate. The bulky nucleotide was introduced into intact cells through digitonin permeabilization and was used by the mutant kinase to thiophosphorylate direct targets [51, 52]. The thiophosphorylated substrates were then immunoprecipitated using a thiophosphate-specific antibody, resulting in identification of 28 new targets [50]. This initial approach did not identify the actual phosphorylation sites, but the known AMPK recognition motif was used to predict the phosphorylation sites on four (PPP1R12C, BAIAP2, CDC27 and PAK2), which were subsequently independently validated [50].

Since existing AMPK recognition motifs were used in this approach to identify the likely sites, sites that did not adhere to these motifs would have been missed. Another drawback was that it might identify as false positives proteins that co-precipitated with the thiophosphorylated substrates. A recent refinement of this method allows concurrent identification of both the substrate and its thiophosphorylation site(s): the thiophosphorylated proteins are digested with trypsin and the

modified peptides isolated via a peptide capture approach, allowing the sites to be directly identified by tandem mass spectrometry [53]. When applied to AMPK (Fig. 5C) [54], the 32 phosphorylation sites (27 of them novel) most frequently identified across replicate screens showed striking resemblance to the established AMPK phosphorylation motif [43, 45, 46, 48]. Hydrophobic residues (L, M, I) were observed at P-5, basic residues (R>K), usually at P-3 but also occasionally at P-4 or P-2, neutral polar residues (S, N, T, Q) at P-2, polar and/or charged residues (S, D, N) at P+3, and hydrophobic residues (usually L) at P+4 [54]. As suggested by an earlier approach [46], a subset of sites contained basic residues at P-6, although they were not present at all sites. Some of the novel sites [54], as well as many established AMPK target sites (see supplementary Table 1 for an updated list of 64 well-validated AMPK target sites, and Fig. 6), contain proline at P+2, which is not selected in screens utilizing cell-free assays [48]. This is most likely due to the functional overlap between AMPK phosphorylation motifs and 14-3-3 binding sites, with the latter usually having a phosphoserine with R at P-3, S at P-2, and P at P+2 [55]. No strong distal motif outside of P-5 to P+4 emerged from analysis of the phosphorylation sites identified in this study [54] or of 64 well-validated AMPK sites (supplementary Table 1), suggesting that the primary recognition motif is contained within this window. It should also be noted that the target sequences listed in supplementary Table 1 that were used to generate Fig. 6 excluded autophosphorylation sites, such as Ser108 on AMPK-β1 [56], Ser491 on AMPK-α2 [57] , and several others [58]. While they can have critical functions, autophosphorylation sites do not always conform to the recognition motif found on other substrates, perhaps because their close proximity to the kinase domain means that this is not necessary.

## Prediction of novel targets using the AMPK recognition motif

AMPK now has a recognition motif (Fig. 6) that is among the best defined of any protein kinase, making it potentially useful for predicting novel targets. It is important to note, however, that there are likely to be many sites that conform to this motif that are not targets for AMPK, either because they are not accessible to the kinase due to some aspect of structure or protein:protein interaction, or because of their subcellular location. Since important phosphorylation sites are likely to be conserved, several groups have searched protein databases to find motifs matching earlier versions of the recognition motif, and then filtered their results by evolutionary conservation. This led to the

identification of phosphorylation sites with important regulatory functions on RAPTOR [48], ULK1 [59], Class IIA histone deacetylases [60], and AMOTL1, the latter involved in Hippo signaling [61]. Since AMPK phosphorylation often triggers 14-3-3 binding, the identification of some of these was aided by screening for candidates that bound 14-3-3 proteins in an AMPK-dependent manner. Recently, a similar analysis was conducted, searching for motifs in the proteome containing basic residues at P-6 and P-4 and hydrophobic residues at P-5 and P+4, and several sites predicted were validated as AMPK targets in cell-free assays [62]. In a parallel approach, an antibody recognizing the core recognition motif (LxRxxpS/pT) was used to immunoprecipitate proteins following AMPK activation in hepatocytes, which were then identified by mass spectrometry. The majority of the proteins identified did contain that motif, and several of these were validated as AMPK targets [63].

These successes suggest that it should be possible to explore the full network of AMPK substrates, and several resources for this are available. The motif prediction platform Scansite [64] (http://scansite.mit.edu) provides a position weight matrix (PWM)-based algorithm for querying a single protein, or a predefined protein database, for optimal AMPK motifs. Building on this, two of us [54] developed an AMPK motif prediction platform (available at https://github.com/BrunetLabAMPK/AMPK_motif_analyzer) by constructing a PWM from a list of substrates, which contains the frequencies of each amino acid at each position in the motif from P-5 to P+4, and controls for a background of general phosphorylation motifs. The platform allows users to upload tab-delimited files, and the output retains all data associated with each scored site. Thus, large quantitative phosphoproteomic datasets can be queried for AMPK-like sites. The PWM can also be used independently in other web-accessible platforms, such as FIMO [65] or Scansite to query entire proteomes or other databases. In addition, the consensus motif may be used to identify the likely AMPK phosphorylation sites on the many proteins that have been identified as in substrate screens [50, 66] or as AMPK interacting proteins, as some of the latter may also be direct AMPK targets [67, 68].

Finally, it might be worth noting that AMPK does seem to tolerate a little more flexibility in its recognition motif than many other protein kinases. Thus, it will accept histidine rather than arginine or lysine as the basic residue at P-3 or P-4, which many other “Basophilic” kinases do not. Moreover, it appears to accept a little slippage in the exact position of the hydrophobic residue at P-

293 5. Thus, in NOS1, NOS3, PAK2 and PFKFB2 the hydrophobic residue is at P-4 rather than P-5,
294 although in all these cases it is followed by an arginine at P-3 (Supplementary Table 1).

## 295 Concluding remarks

296 Some important unresolved issues regarding the inputs and outputs impinging on AMPK are
297 outlined in the "Outstanding questions" Box. AMPK is now known to be activated through a
298 variety of upstream inputs, including the canonical mechanism involving sensing of adenine
299 nucleotide ratios, and non-canonical mechanisms such as those used by compounds that bind at the
300 ADaM site, and the $Ca^{2+}$-dependent pathway involving CaMKKβ.

301 The number of outputs, i.e. identified downstream targets, is also expanding rapidly. The
302 availability of phosphorylation site prediction tools should greatly assist in elucidation of the
303 complete network of downstream targets. However, the current site prediction tools do have some
304 limitations. AMPK-α1 and -α2 lie in the same branch of the kinome as up to twelve AMPK-related
305 kinases, and some of the latter, notably the MARKs [69], have similar recognition motifs. Thus,
306 using the AMPK recognition motif to predict sites may also yield targets for AMPK-related kinases.
307 Covalent modifications within the proximity of the target site (such as oxidation of methionine
308 when this is the hydrophobic residue at P-5 [70]) might also affect recognition. In addition,
309 sequences and/or higher order structures outside of the P-5 to P+4 window, such as the amphipathic
310 helix from P-15 to P-5 upstream of Ser79 on ACC1, can enhance the recognition of specific sites.
311 Because these are not essential, the increased power they could bring to site prediction is currently
312 lost. One future direction might be to investigate whether multiple subsets of distal recognition
313 motifs exist, which might contain elements that increase predictive power. Further exploration of
314 the network in different contexts, for example when activated by different inputs, will be a key step
315 in better understanding the physiological and pathological roles of this central energy-sensing
316 kinase.

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324

## FIGURE LEGENDS

**Figure 1: Canonical mechanism of activation of AMPK by adenine nucleotides, and the $Ca^{2+}$-dependent mechanism mediated by CaMKKβ.** AMP binding activates AMPK by three effects, i.e. promotion of Thr172 phosphorylation by LKB1 (effect #1), inhibition of Thr172 dephosphorylation by protein phosphatases (PP) (effect #2), and allosteric activation (effect #3). All three effects are opposed by binding of ATP, while binding of ADP mimics effect #2 and #1, but not #3. CaMKKβ phosphorylates the same site as LKB1 (Thr172) in response to increases in cellular $Ca^{2+}$.

**Figure 2: Crystal structure of the human α1β2γ1 heterotrimer in complex with β-cyclodextrin, staurosporine, and AMP, with Thr172 phosphorylated.** Atomic co-ordinates are from the PDB file 4RER [8]. The model was rendered in PyMOL v1.7.4.2 with the majority of the polypeptide in "cartoon" view and the α-linker in "sphere" view. The domains referred to in the text are color coded and labeled. The kinase inhibitor staurosporine in the active site, and the side chain of phospho-Thr172, are in "sphere" view, and β-cyclodextrin in the glycogen-binding site of the β-CBM in "stick" view, all with C atoms in green, O red, and N blue (H omitted). The curved dotted line in the center shows the approximate boundary between the "catalytic module" (containing the α-KD and β-CBM) and the "nucleotide-binding module" (containing the γ subunit and the C-terminal domains of α and β); the α-AID and α-linker form one of the flexible connectors linking these two modules. Note how the α-RIM2 section of the α-linker (in magenta) contacts site 3 of the γ subunit with its bound AMP.

**Figure 3: Structures of the kinase domain (α-KD) and auto-inhibitory domains (α-AID) of the α subunit in (A) inactive and (B) active conformations.** Atomic co-ordinates are from the PDB files 4RED (A) and 4RER (B) [8], with only the α-KD, α-AID and the start of the α–linker being displayed in (B). The color-coding of domains is as in Fig. 2. Most of the structures are rendered in "cartoon" view (PyMOL v1.7.4.2), but the side chains of the regulatory spine [18] (Leu81, white; Leu70, red; Phe160, magenta; His139, blue), and phosphorylated Thr172 in (B), are in "sphere" view. Note how the residues of the regulatory spine are stacked in alignment in (B) but not in (A). In (A), the α-AID shown is that attached to the other molecule of α-KD:α-AID within the crystal dimer, but in solution the α-AID from the same molecule is thought to adopt this position [8].

**Figure 4: Selection of AMPK activating compounds grouped according to their mechanisms of action.** (A) pro-drugs converted into AMP analogs by cellular enzymes; (B) compounds that bind at the ADaM site; (C) compounds that act by inhibiting mitochondrial ATP synthesis and thus increase cellular AMP and ADP; (D) antifolate drugs that activate AMPK by inhibiting AICAR transformylase in the pathway of purine nucleotide biosynthesis (shown), thus increasing cellular ZMP. Figure modified from Fig. 1 in [71].

**Figure 5: Approaches used to characterize the AMPK recognition motif. (A) Hypothesis-driven approach using mutations.** Constructs containing 34 residues around Ser79 on ACC1, with and without the indicated mutations, were expressed in bacteria and phosphorylated by AMPK in cell-free assays. Changes in kinetic parameters ($k_{cat}/K_m$) for each mutant relative to the wild type are represented by the lengths of the bars above (increases) or below (decreases) the indicated amino acid [46]. **(B) Positional scanning peptide library.** Phosphorylation by AMPK in cell-free assays of peptide mixtures (10-mers) containing serine and threonine at position 6, a fixed amino acid at one position (e.g. P-5, illustrated), and random mixtures at all others, revealed preferences for specific amino acids at each position [48]; "x", any amino acid. **(C) Direct thiophosphorylation with gatekeeper mutation.** An AMPK mutant uses a bulky derivative of ATP-γ–S (A*TPγS) to thiophosphorylate direct targets in permeabilized cells [50]. Many direct AMPK phosphorylation sites were identified through isolation and identification by tandem mass spectrometry of the thiophosphorylated peptides [54]. Hydrophobic amino acids are in green, neutral polar in blue, acidic in orange, and basic in red.

**Figure 6: AMPK recognizes a well-defined phosphorylation motif.** The logo motif of 64 known AMPK phosphorylation sites from P-7 to P+7 is presented, and points of interest in the AMPK recognition motif are noted. Color scheme as in Figure 5. The logo motif was generated by WebLogo [72], using a previously described list of 50 well-validated phosphorylation sites [54] updated with 14 additional sites from the literature. This full list of 64 well-validated AMPK targets is provided as supplementary Table 1. Human sequences were used to generate this motif.

***Glossary:***

**α-AID:** auto-inhibitory domain, the domain that follows the kinase domain in AMPK-α subunits, and which inhibits the kinase domain in the absence of AMP

**α-KD:** kinase domains on the α subunits of AMPK, which are related to the catalytic domains in other members of the "eukaryotic" protein kinase (ePK) family

**α-linker:** region of the AMPK-α subunit that connects the α-AID to the C-terminal domain, important in the mechanism of regulation by AMP

**α-RIM1/α-RIM2:** conserved sequences within the α-linker, which interact with the AMPK-γ subunit when AMP is bound at site 3

**ADaM site:** the "Allosteric Drug and Metabolite" binding pocket, located between the α subunit kinase domain and the β subunit carbohydrate-binding module, where drugs such as A-769662 bind, and where naturally occurring metabolites are speculated to bind

**CBS repeat:** a sequence motif, first found in the enzyme cystathionine β-synthase, that always occurs as tandem repeats; a single twin repeat often binds a ligand containing adenosine, such as AMP, ATP or S-adenosylmethionine

**kinase recognition motif:** amino acid sequence surrounding a phosphorylation site, which promotes a given protein kinase to target that residue

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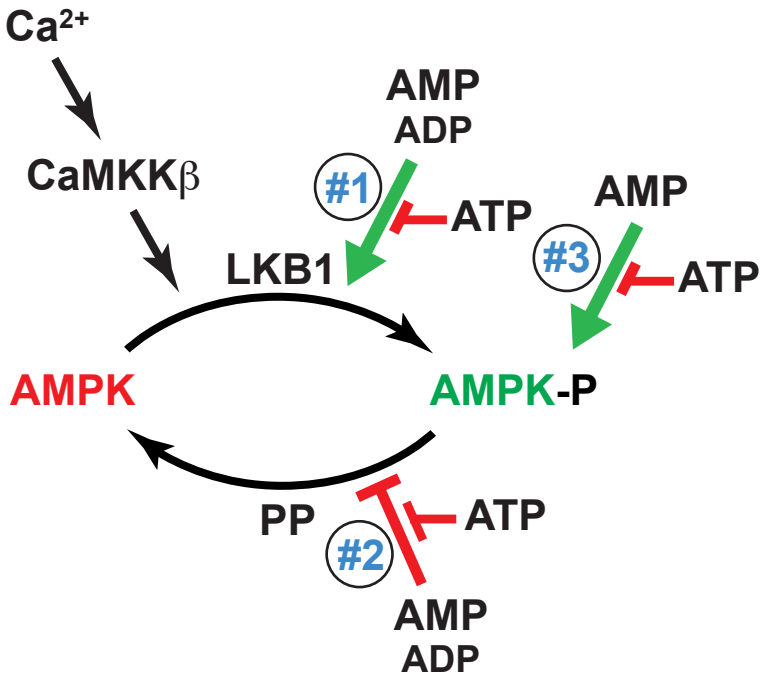
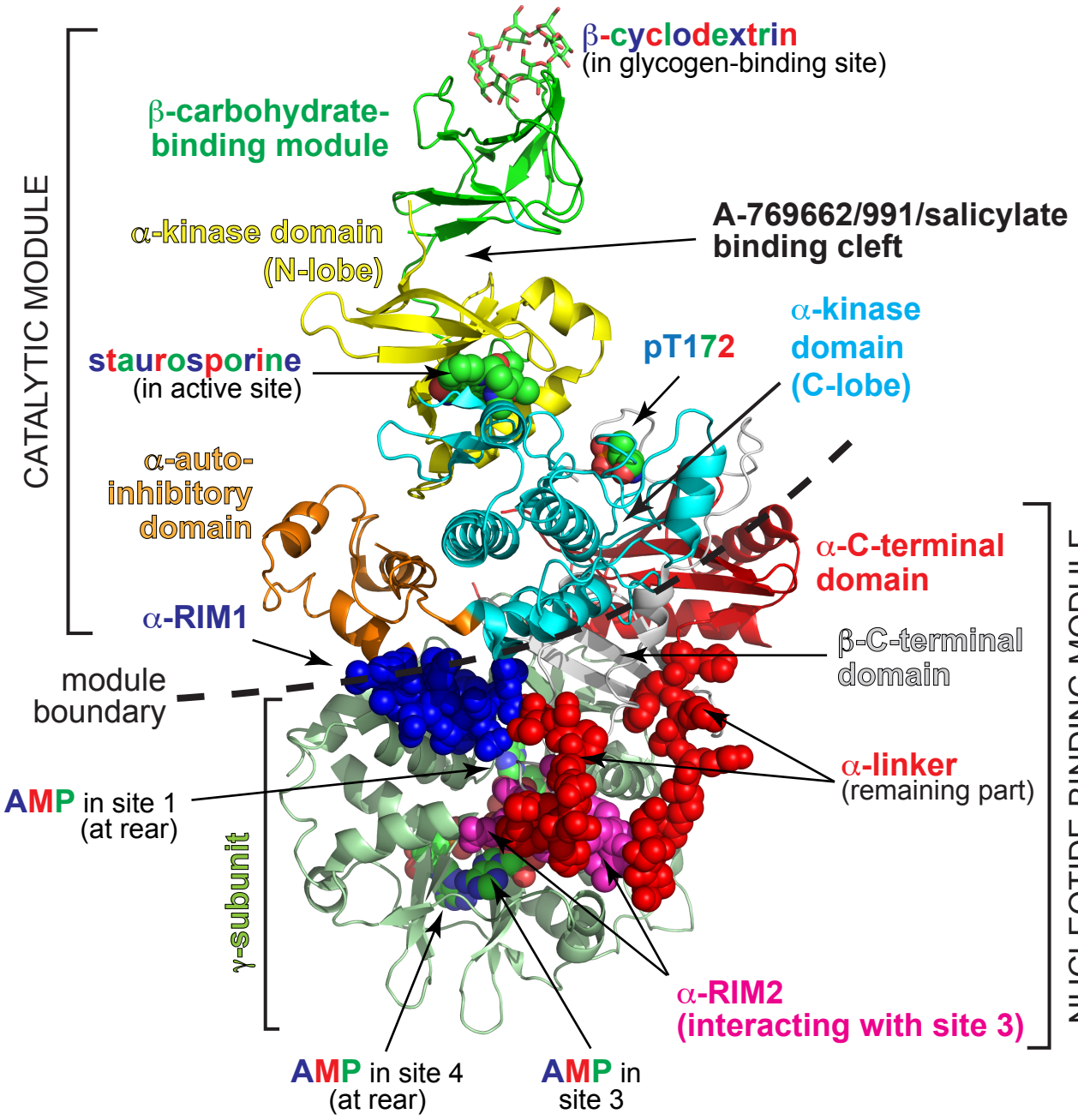
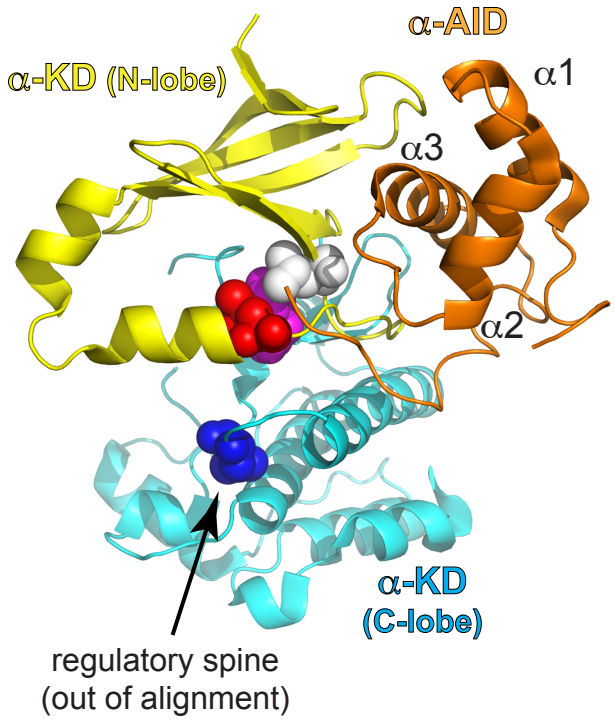
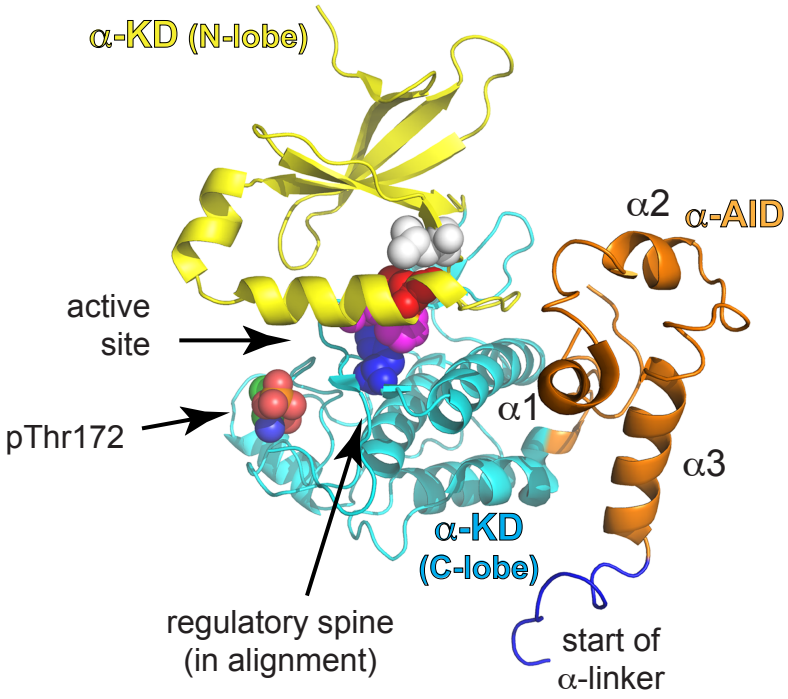

Ca2+
CaMKKβ
LKB1
AMP
ADP
#1
ATP
AMP
#3
ATP
AMPK
AMPK-P
PP
#2
ATP
AMP
ADP


β-cyclodextrin
(in glycogen-binding site)
β-carbohydrate-binding module
α-kinase domain (N-lobe)
A-769662/991/salicylate binding cleft
α-kinase domain (C-lobe)
pT172
staurosporine
(in active site)
α-auto-inhibitory domain
α-C-terminal domain
α-RIM1
β-C-terminal domain
module boundary
α-linker
(remaining part)
AMP in site 1
(at rear)
γ-subunit
α-RIM2
(interacting with site 3)
AMP in site 4
(at rear)
AMP in site 3
CATALYTIC MODULE
NUCLEOTIDE-BINDING MODULE

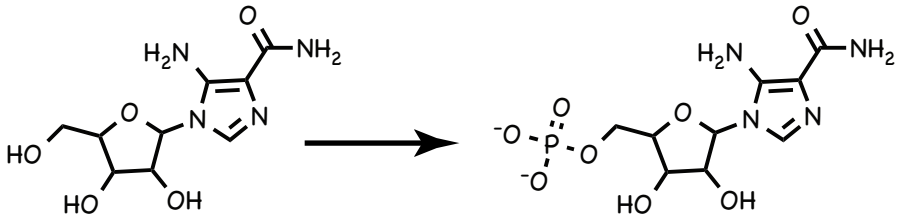
A) α-KD:AID (inactive conformation)



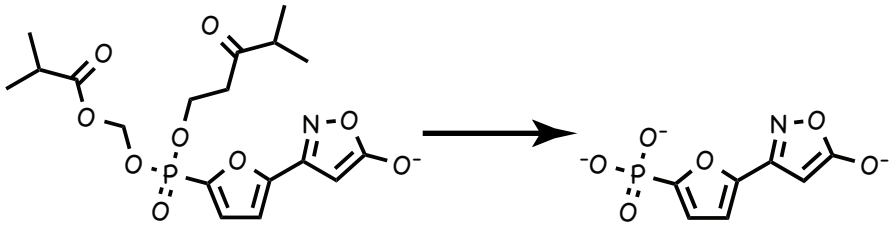

B) α-KD:AID (active conformation)



A) Pro-drugs converted within cells to AMP analogs

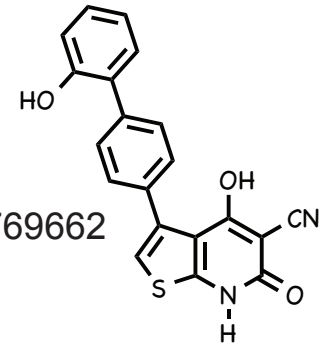


AICAR ZMP

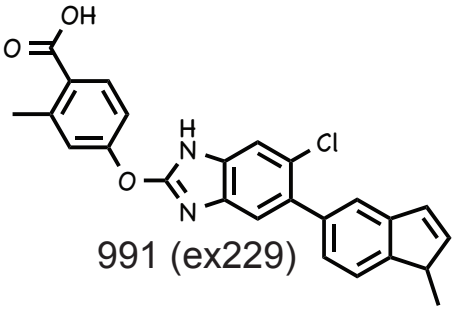


C13 C2

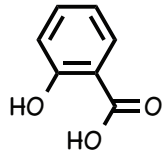
B) Compounds that bind between α-KD and β-CBM



A-769662

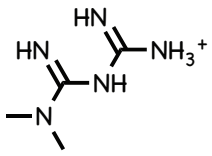


991 (ex229)

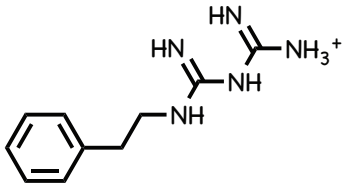


salicylate

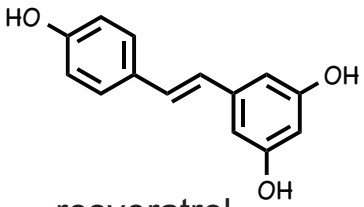
C) Compounds that inhibit mitochondrial ATP synthesis and increase cellular AMP



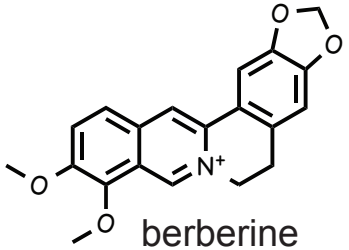
metformin



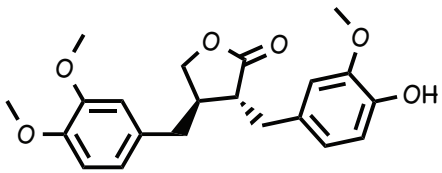
phenformin



resveratrol

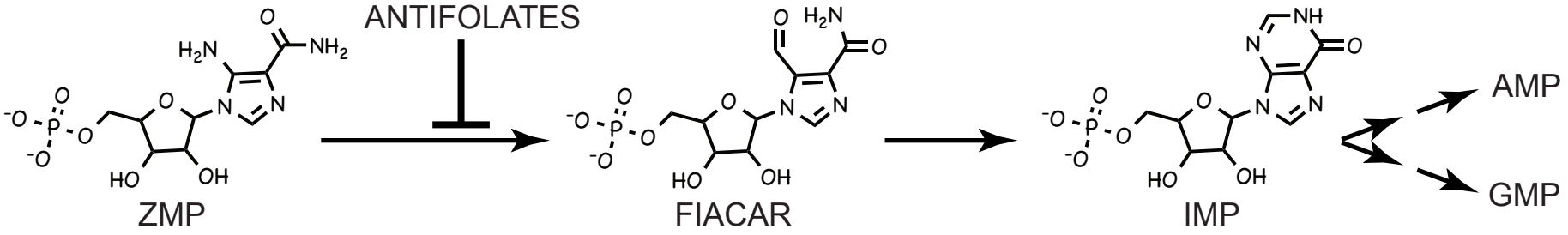


berberine



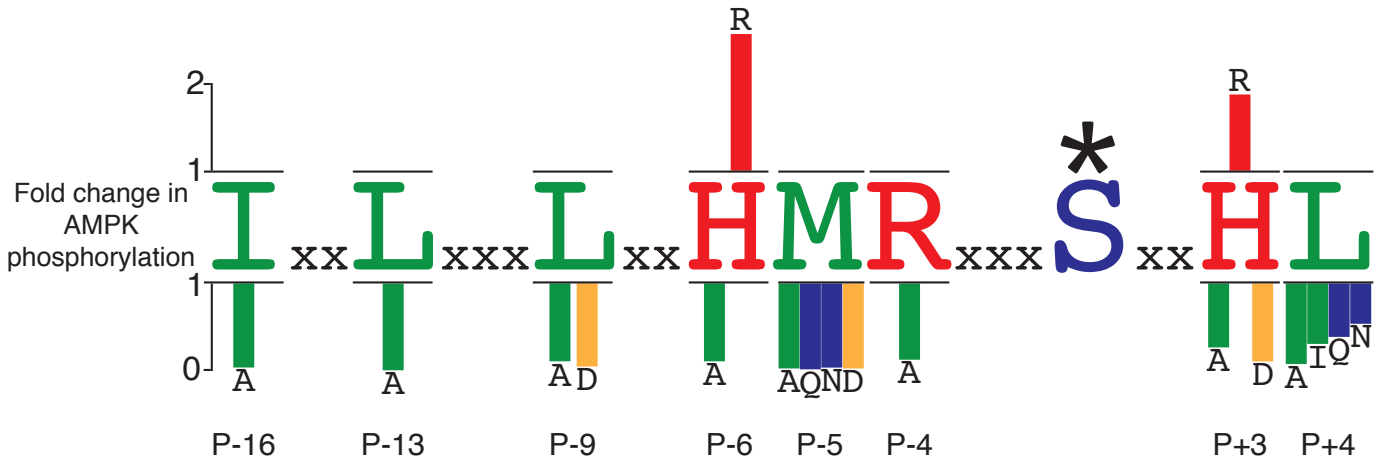
arctigenin

D) Mechanism by which antifolates (e.g. pemetrexed, methotrexate) cause ZMP accumulation:



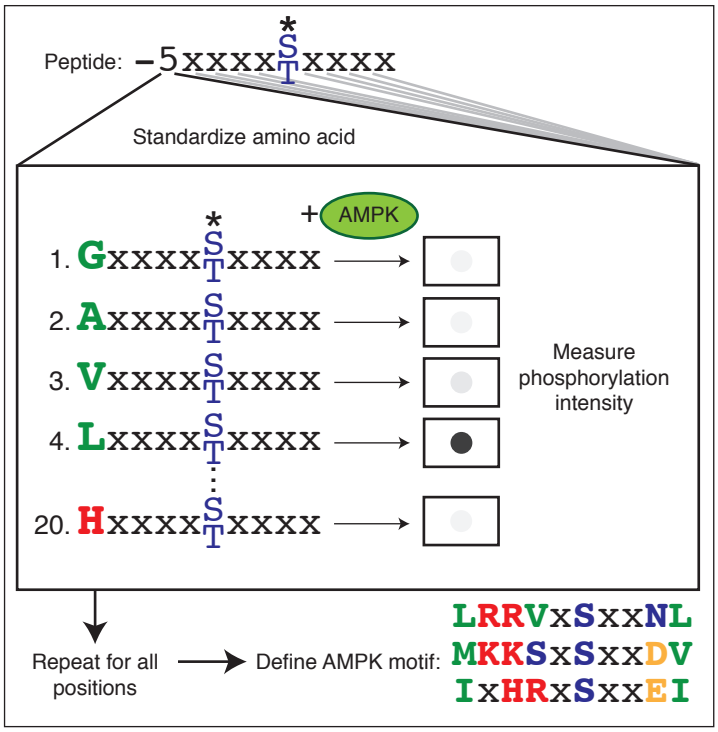

ZMP FIACAR IMP

A. Hypothesis-driven mutations



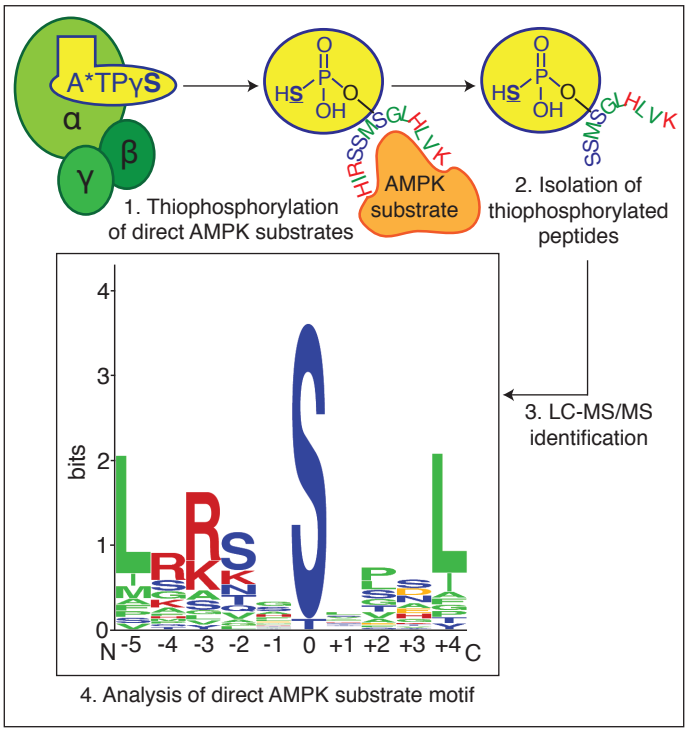

adapted from Scott et al, 2002

B. Positional scanning peptide library




adapted from Gwinn et al, 2008

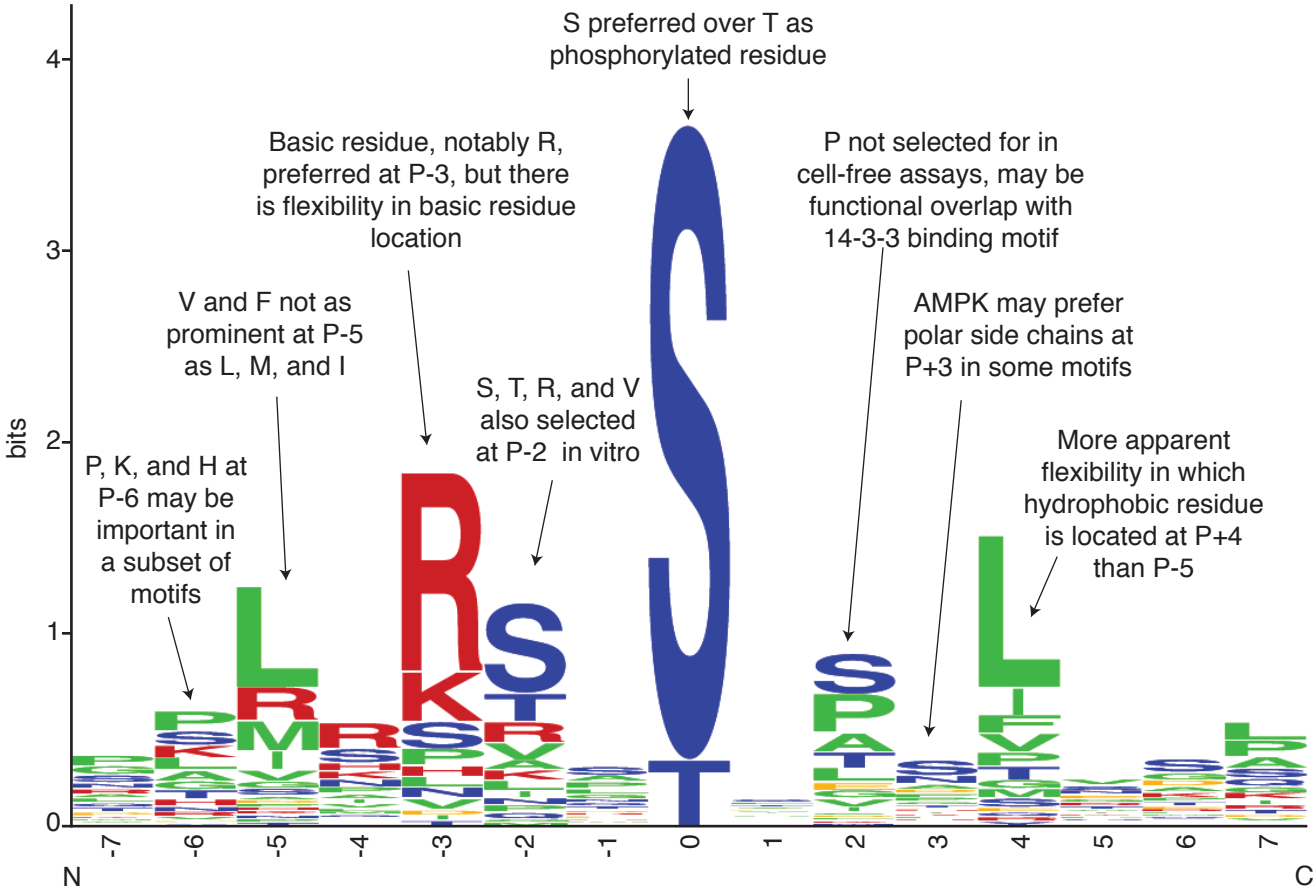
C. Direct thiophosphorylation of substrates




adapted from Banko et al, 2011 and Schaffer et al, 2015

Figure 6

A


S preferred over T as phosphorylated residue
Basic residue, notably R, preferred at P-3, but there is flexibility in basic residue location
P not selected for in cell-free assays, may be functional overlap with 14-3-3 binding motif
V and F not as prominent at P-5 as L, M, and I
AMPK may prefer polar side chains at P+3 in some motifs
S, T, R, and V also selected at P-2 in vitro
P, K, and H at P-6 may be important in a subset of motifs
More apparent flexibility in which hydrophobic residue is located at P+4 than P-5
bits
0
1
2
3
4
-7
-6
-5
-4
-3
-2
-1
0
1
2
3
4
5
6
7
N
C

## Supplementary Material

# AMPK: an energy-sensing pathway with multiple inputs and outputs

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**Table S1: List of 64 validated sites phosphorylated by AMPK.**

| Short name | Long name | UNIPROT | Site[a] | Motif (± 10 residues)[b] | Criteria[c] | | | | Refs |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | 1[d] | 2[e] | 3[f] | 4[g] | |
| ACACA | Acetyl-CoA carboxylase 1 | Q13085 | S80 | GLALHIRSSMSGLHLVKQGRD | X | X | | X | [3-6] |
| ACACB | Acetyl-CoA carboxylase 2 | O00763 | S222 | TRVPTMRPSMSGLHLVKRGRE | X | X | | | [7-10] |
| AMOTL1 | Angiomotin-like protein 1 | Q8IY63 | S793 | TDSSSLRPARSVPSIAAATGT | X | X | | | [11] |
| BAIAP2 | Brain angiogenesis inhibitor 1-associated protein 2 | Q9UQB8 | S366 | TENKTLPRSSSMAAGLERNGR | X | | X | X | [1] |
| BRAF | Serine/threonine-protein kinase B-raf | P15056 | S729 | RSLPKIHRSASEPSLNRAGFQ | X | X | | | [12] |
| CDC27 | Cell division cycle protein 27 homolog | P30260 | S379 | SPPNALPRRSSRLFTSDSSTT | X | | X | | [1] |
| CDC42EP1 | Cdc42 effector protein 1 | Q00587 | S192 | PSEPGLRRSDSLLSFRLDLDL | | | X | X | [2] |
| CDKN1B | Cyclin-dependent kinase inhibitor 1B | P46527 | T198 | PKKPGLRRRQT | X | X | | | [13] |
| CLIP-170 | CAP-Gly domain-containing linker protein 1 | P30622 | S312 | TTSASLKRSPSASSLSSMSSV | X | X | | | [14] |
| CRTC2 | CREB-regulated transcription coactivator 2 | Q53ET0 | S171 | RLPSALNRTSSDSALHTSVMN | X | X | | | [15-18] |
| CRY1 | clock component cryptochrome 1 | Q16526 | S71 | DLDANLRHLNSRLFVIRGQPA | X | X | | | [19] |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| EEF2K | eukaryotic elongation factor 2 kinase | O00418 | S398 | VTFDSLPSSPSSATPHSQKLD | X | X | | | [20] |
| EP300 | Histone acetyltransferase p300 | Q09472 | S89 | KQLSELLRSGSSPNLNMGVGG | X | X | | | [21, 22] |
| FOXO3a | Forkhead box protein O3 | O43524 | S413 | PTGGLMQRSSSFPYTTKGSGL | X | X | | | [23] |
| FOXO3a | Forkhead box protein O3 | O43524 | S588 | QSMQTLSDSLSGSSLYSTSAN | X | X | | | [23] |
| GABABR2 | GABA B receptor R2 subunit | O75899 | S784 | STSVTSVNQASTSRLEGLQSE | X | X | | | [24] |
| GBF1 | Golgi-specific brefeldin A-resistance GEF-1 | Q92538 | T1337 | GRPGKIHRSASDADVVNSGWL | X | X | | | [25] |
| GFPT1 | Glutamine-fructose-6-phosphate aminotransferase-1 | Q06210 | S261 | KGSCNLSRVDSTTCLFPVEEK | X | X | | | [26, 27] |
| GLI1 | Zinc finger protein GLI1 | P08151 | S102 | SLDLQTVIRTSPSSLVAFINS | X | X | | | [28] |
| GLI1 | Zinc finger protein GLI1 | P08151 | S408 | RGDGPLPRAPSISTVEPKRER | X | X | | | [28] |
| GLI1 | Zinc finger protein GLI1 | P08151 | T1074 | SHDQRGSSGHSPPPSGPPNMA | X | X | | | [28] |
| GYS1 | Glycogen Synthase 1 (muscle isoform) | P13807 | S8 | MPLNRTLSMSSLPGLEDW | X | X | | | [29, 30] |
| GYS2 | Glycogen Synthase 2 (liver isoform) | P54840 | S8 | MLRGRSLSVTSLGGLPQW | X | X | | | [31] |
| H2B | Histone H2B | Q16778 | S37 | GKKRKRSRKESYSIYVYKVLK | X | X | | | [32] |
| HDAC5 | Histone Deacetylase 5 | Q9UQL6 | S259 | RDDFPLRKTASEPNLKVRSRL | X | X | | | [33] |
| HDAC5 | Histone Deacetylase 5 | Q9UQL6 | S498 | PRHRPLSRTQSSPLPQSPQAL | X | X | | | [33] |
| HMGCR | 3-hydroxy-3-methylglutaryl-coenzyme A reductase | P04035 | S872 | LVKSHMIHNRSKINLQDLQGA | X | | | | [34-36] |
| IRS1 | insulin receptor substrate 1 | P35568 | S794 | ARHQHLRLSTSSGRLLYAATA | X | X | | | [37, 38] |
| KCNB1 | Potassium voltage-gated channel subfamily B member 1 | Q14721 | S444 | EALERAKRNGSIVSMNMKDAF | X | X | | | [39] |
| KCNB1 | Potassium voltage-gated channel subfamily B member 1 | Q14721 | S541 | DMYNKMAKTQSQPILNTKESA | X | X | | | [39] |
| KLC2 | Kinesin light chain 2 | Q9H0B6 | S545 | DGSGSLRRSGSFGKLRDALRR | X | | | X | [40, 41] |
| LIPE | Hormone-sensitive lipase | Q05469 | S855 | PIAEPMRRSVSEAALAQPQGP | X | | | | [42-44] |
| MAPT | Microtubule-associated protein tau | P10636 | S579 | DLKNVKSKIGSTENLKHQPGG | X | X | | | [45, 46] |
| MDM4 | Mdm4 | O15151 | S342 | SDCSKLTHSLSTSDITAIPEK | X | X | | | [47] |
| NET1 | Neuroepithelial cell-transforming gene 1 protein | Q7Z628 | S100 | KRVRPLARVTSLANLISPVRN | | X | X | X | [2] |
| NOS1 | Neuronal nitric oxide synthase (nNOS) | P29475 | S1417 | YEVTNRLRSESIAFIEESKKD | | X | | | [8, 48] |
| NOS3 | Endothelial nitric oxide synthase (eNOS) | P29474 | S1177 | QEVTSRIRTQSFSLQERQLRG | X | | | | [49] |
| PAK2 | Serine/threonine-protein kinase PAK 2 | Q13177 | S20 | KPPAPPVRMSSTIFSTGGKDP | X | X | X | | [1] |
| PEA15 | Astrocytic phosphoprotein PEA-15 | Q15121 | S116 | KKYKDIIRQPSEEEIIKLAPP | X | X | | | [50] |
| PFKFB2 | 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2 | O60825 | S466 | NQTPVRMRRNSFTPLSSSNTI | X | X | | | [51] |
| PFKFB3 | 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 | Q16875 | S461 | KGPNPLMRRNSVTPLASPEPT | X | X | | | [52, 53] |
| PGC1A | PPAR-gamma coactivator 1-alpha | Q9UBK2 | T178 | NHANHNHRIRTNPAIVKTENS | X | X | | | [54] |
| PGC1A | PPAR-gamma coactivator 1-alpha | Q9UBK2 | S539 | QSYSLFNVSPSCSSFNSPCRD | X | X | | | [54] |
| PIKFYVE | 1-phosphatidylinositol 3-phosphate 5-kinase | Q9Y2I7 | S307 | GKSPARNRSASITNLSLDRSG | X | X | | | [55] |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| PLD1 | Phospholipase D1 | Q13393 | S505 | TDVGSVKRVTSGPSLGSLPPA | X | X | | | [56] |
| PPP1R12C | Protein phosphatase 1 regulatory subunit 12C | Q9BZL4 | S452 | APGAGLQRSASSSWLEGTSTQ | X | X | X | | [1] |
| PRKCQ | protein kinase C-theta | Q04759 | T538 | ENMLGDAKTNTFCGTPDYIAP | X | X | | | [57] |
| RAG1 | V(D)J recombination-activating protein 1 | P15918 | S531 | EWQPPLKNVSSSTDVGIIDGL | X | X | | | [58] |
| RPTOR | Regulatory-associated protein of mTOR | Q8N122 | S792 | ETIDKMRRASSYSSLNSLIGV | X | X | | | [59] |
| RPTOR | Regulatory-associated protein of mTOR | Q8N122 | S722 | PCTPRLRSVSSYGNIRAVATA | | | | X | [59] |
| RRN3 | RNA polymerase I-specific transcription initiation factor | Q9NYV6 | S635 | SSFDTHFRSPSSSVGSPPVLY | X | X | | | [60] |
| SNX17 | Sorting nexin-17 | Q15036 | S437 | ESMVKLSSKLSAVSLRGIGSP | | X | X | X | [2] |
| SREBF1 | Sterol regulatory element-binding protein 1 | P36956 | S396 | SLRTAVHKSKSLKDLVSACGS | X | X | | | [61] |
| TBC1D1 | TBC1 domain family member 1 | Q86TI0 | S237 | PVRRPMRKSFSQPGLRSLAFR | X | X | | X | [62] |
| TBC1D4 | TBC1 domain family member 4 | O60343 | S704 | SSLPSLHTSFSAPSFTAPSFL | X | X | | | [63] |
| TNNI3 | Troponin I, cardiac muscle | P19429 | S150 | KRPTLRRVRISADAMMQALLG | X | X | | | [64, 65] |
| TP53 | p53 | P04637 | S15 | QSDPSVEPPLSQETFSDLWKL | X | X | | | [66, 67] |
| TP73 | Tumor protein p73 | O15350 | S426 | KVHGGMNKLPSVNQLVGQPPP | X | | | | [68] |
| TSC2 | tuberous sclerosis complex 2 | P49815 | S1387 | QPSQPLSKSSSSPELQTLQDI | X | X | | | [69] |
| TXNIP | Thioredoxin-interacting protein | Q9H3M7 | S308 | SRSGLSSRTSSMASRTSSEMS | X | X | | | [70] |
| ULK1 | Serine/threonine-protein kinase ULK1 | O75385 | S467 | PRSSAIRRSGSTSPLGFARAS | X | X | | | [71] |
| ULK1 | Serine/threonine-protein kinase ULK1 | O75385 | S556 | RTSGLGCRLHSAPNLSDLHVV | X | X | | | [71] |
| VASP | Vasodilator-stimulated phosphoprotein | P50552 | T278 | NAMLARRRKATQVGEKTPKDE | X | X | | | [72] |
| YAP1 | Transcriptional coactivator YAP1 | P46937 | S94 | VPMRLRKLPDSFFKPPEPKSH | X | X | | | [73] |

[a]Phosphorylation site (human sequence numbering)

[b]Phosphorylation site motif ±10 residues upstream and downstream of the phosphoamino acid

Red = phosphorylated serine/threonine; brown = bulky hydrophobic residues ((L, I, M, F, V)) at P-5 and P+4; and blue = basic residues at P-6, P-4, P-3 or P-2. In some cases, N-terminal hydrophobic and basic residues are shifted one residue from these standard locations (see main text); these are also color coded. Where no residues are shown, the site is near the N- or C-terminus of the protein.

[c]Criteria that were used to validate AMPK substrates. In general, a site can be considered a well-validated AMPK substrate when both a direct phosphorylation in cell-free assays and AMPK-dependent phosphorylation in intact cells have been demonstrated in a site-specific manner (criteria 1

and 2 together), or the site was identified and validated using the ATP-analog-specific approach in intact, permeabilized cells. In this list, we have generally not taken into account evidence obtained using functional changes in the target protein. Given the size of the AMPK literature, we did not include all sites that have been exclusively validated in cell-free assays, and we may have missed some key substrates or data relevant to the validation of some listed substrates.

[d]cell-free assays demonstrating that AMPK directly phosphorylates the site in question (e.g. via mass spectrometry, $^{32}P$ incorporation or phosphospecific antibodies, preferably with comparisons of phosphorylation with and without mutation at the site)

[e]Demonstration that AMPK phosphorylates the site in intact cells through either; (i) increases in phosphorylation in response to treatment with specific AMPK activators (e.g. A769662, AICAR, 991); (ii) decreases in phosphorylation following knockout or knock-down (e.g. by expression of dominant negative AMPK mutant or interfering RNA); or (iii) the use of less specific AMPK activators (e.g. 2-deoxyglucose, metformin, muscle contraction) in combination with AMPK knockdown or inhibition (e.g. using compound C, although the authors discourage the use of that compound, which is a very non-selective kinase inhibitor).

[f]Demonstration that ATP analog-specific (AS) AMPK thiophosphorylates the specific site in intact permeabilized cells [1].

[g]Identification of the site with high confidence in Schaffer et al [2].

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***Glossary:***

**α-AID:** auto-inhibitory domain, the domain that follows the kinase domain in AMPK-α subunits, and which inhibits the kinase domain in the absence of AMP

**α-KD:** kinase domains on the α subunits of AMPK, which are related to the catalytic domains in other members of the "eukaryotic" protein kinase (ePK) family

**α-linker:** region of the AMPK-α subunit that connects the α-AID to the C-terminal domain, important in the mechanism of regulation by AMP

**α-RIM1/α-RIM2:** conserved sequences within the α-linker, which interact with the AMPK-γ subunit when AMP is bound at site 3

**CBS repeat:** a sequence motif, first found in the enzyme cystathionine β-synthase, that always occurs as two tandem repeats; a single twin repeat often binds a ligand containing adenosine, such as AMP, ATP or S-adenosylmethionine

**kinase recognition motif:** amino acid sequence surrounding a phosphorylation site, which promotes a given protein kinase to target that residue

**TRENDS:**

- AMPK is an energy-sensing protein kinase activated by phosphorylation of Thr172 within its catalytic α subunit. It binds AMP and/or ADP, both signals of energy stress, via its regulatory γ subunit. This activates the kinase by promoting Thr172 phosphorylation, inhibiting Thr172 dephosphorylation and allosteric activation.
- AMP binding to the γ subunit causes its interaction with the "α-linker" region of the α subunit. This pulls the auto-inhibitory domain on the α subunit away from the kinase domain, triggering activation.
- AMPK is also activated by binding of synthetic and naturally occurring drug-like molecules that bind in the allosteric drug and metabolite (ADaM) site, between the α and β subunits
- AMPK has a well-defined recognition motif that has been established both by hypothesis-driven approaches and by various unbiased screens. It now has over 60 well-validated substrates.

***Outstanding questions:***

1. Given that site 3 on the AMPK-γ subunit seems to be the crucial site where binding of AMP causes activation, what is the function of AMP binding at the other two sites, sites 1 and 4?
2. How does binding of AMP or ADP to the AMPK-γ subunit promote phosphorylation of the AMPK-α subunit at Thr172 by upstream kinases such as LKB1?
3. Are there any naturally occurring metabolites in mammalian cells that bind to the allosteric drug and metabolite (ADaM) binding pocket between the α and β subunits, and thus activate or inhibit AMPK?
4. How are responses to different inputs affected by the numerous post-translational modifications that have been reported to occur on each AMPK subunit?
5. Does AMPK phosphorylate a different subset of substrates, depending on the inputs via which it was activated?
6. Are there subsets of primary or secondary structures, beyond the defined window from amino acids -5 to +4 in the AMPK recognition motif, which can help better predict substrates?
7. How much flexibility is there in the recognition motif surrounding any given AMPK phosphorylation site? For example can more distal features, such as the N-terminal amphipathic α-helices that are observed in acetyl-CoA carboxylase and HMG-CoA reductase, determine how rigidly the motif from P-5 to P+4 must adhere to the canonical recognition motif?